FORM PTO-1390 (REV. 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		B0192/7031
		U S APPLICATION NO (If known, see 37 CFR 1 5) 09/890229
INTERNATIONAL APPLICATION NO. PCT/GB00/00263	INTERNATIONAL FILING DATE 28 January 2000 (28.01.00)	PRIORITY DATE CLAIMED 28 January 1999 (28.01.99)

TITLE OF INVENTION
MANIPULATING ISOPRENOID EXPRESSION

APPLICANT(S) FOR DO/EO/US
BRAMLEY, Peter Michael; HARKER, Mark

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the earliest claimed priority date (PCT Article31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are attached hereto (required only if not transmitted by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(C)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C.1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

Copy of PCT/RO/101 form
Copy of PCT Published Application with Search Report
PCT/IB/308 form

Copy of International Preliminary Examination Report
Statement Pursuant to 37 CFR 1.821(f) w/ diskette and paper copy of sequence listing
Express Mail Label No. EL819461955US
Date Mailed: July 27, 2001

JC17 Rec'd PCT/PTO 27 JUL 2001

U.S. APPLICATION NO (if known, see 37 CFR 1.5) 09/890229	INTERNATIONAL APPLICATION PCT/GB00/00263	ATTORNEY'S DOCKET NUMBER B0192/7031

21. ☒ The following fees are submitted:

		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):			
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1000.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$860.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but but international search fee paid to USPTO (37 CFR 1.445(a)(2)).paid to USPTO	$710.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) But all claims did not satisfy provisions of PCT Article 33(1)-(4)	$690.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT = 860.00			
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	34-20 =	14	X $18.00	$252.00	
Independent Claims	6- 3 =	3	X $80.00	$240.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+$270.00	$	
TOTAL OF ABOVE CALCULATIONS			=	$1352.00	
Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$	
SUBTOTAL			=	$1352.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE			=	$1352.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate coversheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED			=	$1352.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 1352.00 To cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ In the amount of $_____ To cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23/2825. A duplicate of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210
Tel: (617) 720-3500

CUSTOMER NUMBER 23628

John R. Van Amsterdam
SIGNATURE

John R. Van Amsterdam
NAME

40,212
REGISTRATION NO

495341.1

JC17 Rec'd PCT/PTO 27 JUL 2001

U.S. APPLICATION NO (If known, see 37 CFR 1.5) 09/890229	INTERNATIONAL APPLICATION PCT/GB00/00263	ATTORNEY'S DOCKET NUMBER B0192/7031

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

		CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1000.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$860.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but but international search fee paid to USPTO (37 CFR 1.445(a)(2)).paid to USPTO	$710.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) But all claims did not satisfy provisions of PCT Article 33(1)-(4)	$690.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT = 860.00		

Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	34-20 =	14	X $18.00	$252.00	
Independent Claims	6- 3 =	3	X $80.00	$240.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+$270.00	$	
TOTAL OF ABOVE CALCULATIONS			=	$1352.00	
Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$	
SUBTOTAL			=	$1352.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE			=	$1352.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate coversheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED			=	$1352.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 1352.00 To cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ In the amount of $_____ To cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23/2825. A duplicate of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO.

WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210
Tel: (617) 720-3500

CUSTOMER NUMBER 23628

John R. Van Amsterdam
SIGNATURE

John R. Van Amsterdam
NAME

40,212
REGISTRATION NO

495341.1

Attorney's Docket No. **B0192/7031**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Bramley et al.
U.S. Serial No.	:	09/890,229
International Application No.	:	PCT/GB00/00263
International Filing Date	:	28 January 2000 (28.01.00)
Earliest Priority Date	:	28 January 1999 (28.01.99)
Title	:	MANIPULATING ISOPRENOID EXPRESSION
Examiner	:	Unknown
Art Unit	:	1651

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail via express mail, addressed to Box PCT, U.S. Patent and Trademark Office, P.O. Box 2327, Arlington, VA 22202, on the 27th day of November, 2001.

Monica E. Zombori

Box PCT
U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

COMMUNICATION

Dear Sir:

The Notification of Missing Requirements which was mailed on September 6, 2001, states that claims 12, 13, and 26 are multiple dependent claims, and as such the multiple dependent claim fee of $270.00 is required for these claims. However, this seems to be an error. Upon review of the application, Applicants found that claims 12, 13, and 26 were not multiple dependent claims. Therefore, Applicants believe there is no additional claim fee required in this application.

If any fee is determined to be required, please charge the balance to the account of the undersigned, deposit account number 23/2825.

Respectfully submitted,

John R. Van Amsterdam, Reg. No. 40,212
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, MA 02210-2211
Tel. (617) 720-3500

Attorney's Docket No.: B0192/7031
Date: November 27, 2001
x12/06/01

577963.1

CRF Errors Corrected by the STIC Systems Branch

Serial Number: 09/870229

CRF Processing Date: 10/05/2001
Edited by: mm
Verified by: _____ (STIC sta

☐ Changed a file from non-ASCII to ASCII

☐ Changed the margins in cases where the sequence text was "wrapped" down to the next line.

☐ Edited a format error in the Current Application Data section, specifically:

_____

☐ Edited the Current Application Data section with the actual current number. The number inputted by the applicant was ☐ the prior application data; or ☐ other _____

ENTERED

☐ Added the mandatory heading and subheadings for "Current Application Data".

☐ Edited the "Number of Sequences" field. The applicant spelled out a number instead of using an integer.

☐ Changed the spelling of a mandatory field (the headings or subheadings), specifically:

_____

☐ Corrected the SEQ ID NO when obviously incorrect. The sequence numbers that were edited were:

_____

☐ Inserted or corrected a nucleic number at the end of a nucleic line. SEQ ID NO's edited:

_____

☐ Corrected subheading placement. All responses must be on the same line as each subheading. If the applicant placed a response below the subheading, this was moved to its appropriate place.

☐ Inserted colons after headings/subheadings. Headings edited included:

_____

☐ Deleted extra, invalid, headings used by an applicant, specifically:

_____

☑ Deleted: ☑ non-ASCII "garbage" at the beginning/end of files; ☐ secretary initials/filename at end of file; ☐ page numbers throughout text; ☐ other invalid text, such as _____

☐ Inserted mandatory headings, specifically: _____

☐ Corrected an obvious error in the response, specifically:

_____

☐ Edited identifiers where upper case is used but lower case is required, or vice versa.

☐ Corrected an error in the Number of Sequences field, specifically:

_____

☐ A "Hard Page Break" code was inserted by the applicant. All occurrences had to be deleted.

☐ Deleted *ending* stop codon in amino acid sequences and adjusted the "(A)Length:" field accordingly (error due to a PatentIn bug). Sequences corrected: _____

☐ Other:

_____

_____

_____

_____

*Examiner: The above corrections must be communicated to the applicant in the first Office Action. DO NOT send a copy of this form.

3/1/95

#3

Attorney's Docket No. **B0192/7031**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Bramley et al.
U.S. Serial No.	:	09/890,229
International Application No.	:	PCT/GB00/00263
International Filing Date	:	28 January 2000 (28.01.00)
Earliest Priority Date	:	28 January 1999 (28.01.99)
Title	:	MANIPULATING ISOPRENOID EXPRESSION
Examiner	:	Unknown
Art Unit	:	1651

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail via express mail, addressed to Box PCT, U.S. Patent and Trademark Office, P.O. Box 2327, Arlington, VA 22202, on the 27th day of November, 2001.

Monica E. Zombori

Box PCT
U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

STATEMENT PURSUANT TO 37 CFR 1.821(f) AND 37 CFR 1.825 (a) and (b)

This statement is made pursuant to 37 CFR 1.821 (f), and 37 CFR 1.825 (a) and (b). Applicants submit herewith a substitute copy of the written sequence listing and a computer readable diskette to comply with the sequence requirements.

Applicants' attorney states that the information recorded in computer readable form is identical to the written sequence listing and that neither the computer readable form nor the written sequence listing contain new matter.

Respectfully submitted,

John R. Van Amsterdam, Reg. No. 40,212
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, MA 02210-2211
Tel. (617) 720-3500

Attorney's Docket No.: B0192/7031
Date: November 27, 2001
x12/06/01

577949.1

Attorney's Docket No: B0192/7031

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Peter BRAMLEY et al.
International Application No.	:	PCT/GB00/00263
International Filing Date	:	28 January 2000 (28.01.00)
Earliest Priority Date	:	28 January 1999 (28.01.99)
Title	:	MANIPULATING ISOPRENOID EXPRESSION

Commissioner for Patents
Box PCT
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the United States national phase application of the above-identified PCT international application as follows.

In the Specification

Please amend the specification as follows:

Please add the following section as the first section of the specification following the title.

Related Applications

This application is a national stage filing under 35 U.S.C. § 371 of PCT International application PCT/GB00/00263, and filed 28 January 2000, which was published under PCT Article 21(2) in English.

Foreign priority benefits are claimed under 35 U.S.C. § 119(a)-(d) of Great Britain application number GB 9901902.8, filed 28 January 1999.

Please substitute paragraphs of the specification as follows.

Substitute the following paragraph for the paragraph that begins on line 33, page 13 of the specification as filed.

Figure 3: is an illustration of an amino acid sequence alignment of *DXP* synthases used in the present invention, Synechocystis sp. 6803 (S.s) (GenBank D90903) (SEQ ID NO: 1), B. subtilis (B.s) (GenBank D84432) (SEQ ID NO: 2) and *E. coli* (E.c) (GenBank AF035440) (SEQ ID NO: 3). The consensus line (consen) shows residues conserved in all three sequences (upper case letters) or residues which are identical in two sequences and replaced by an equivalent amino acid in the third sequence (+). The conserved histidine domain putatively involved in proton transfer is over lined and numbered 1. The second over lined domain (2) denotes the consensus thiamin pyrophosphate (TPP)-binding motif.

Substitute the following paragraph for the paragraph that begins on line 35, page 14 of the specification as filed.

Figure 6: is a diagrammatic illustration of vector pVB6_TSEC_LML (SEQ ID NO: 6).

Substitute the following paragraph for the paragraph that begins on line 3, page 15 of the specification as filed.

Figure 7: is a diagrammatic representation of plasmid pVB6_35S_TSEC_LML (SEQ ID NO: 5).

Substitute the following paragraph for the paragraph that begins on line 6, page 15 of the specification as filed.

Figure 8: is an illustration of the amino acid sequence of *E. coli* DXPS (SEQ ID NO: 3).

Substitute the following paragraph for the paragraph that begins on line 9, page 15 of the specification as filed.

Figure 9: is an illustration of the transit peptide used in tomato plants (SEQ ID NO: 4).

Substitute the following paragraph for the paragraph that begins on line 15, page 16 of the specification as filed.

Based on the nucleotide sequence of ORF s111945 from the genome database for Synechocystis sp. PCC 6803 [23], primers were designed to clone the putative dxps gene by polymerase chain reaction (PCR). The forward primer 5'-GTCCCAATCCACCATGCACATCAG-3' (SEQ ID NO: 7) overlaps the beginning of the coding sequence. The reverse primer 5'-CCCTCGACAAATGCAAAATGTATC-3' (SEQ ID NO: 8) lies outside the stop codon of the gene. A PCR (25 cycles) using Pfu DNA polymerase (Stratagene) produced a DNA fragment of the expected size (~1.9 kb). Subsequent sequencing of the fragment confirmed the product to be the ORF s111945. The B. subtilis dxps gene was also cloned by PCR using primers designed to amplify the gene encoding the product YqiE, identified in the Bacillus subtilis genome database [24]. The forward primer 5'-GATCCGCTATGGATCTTTTATC-3' (SEQ ID NO: 9) contains a modified base substitution at the predicted start codon (underlined) for improved expression in *E. coli*. The reverse primer 5'-ATCTAATCGTTCTTTCTTTGAC-3' (SEQ ID NO: 10) lies outside the stop codon of the dxps gene. After PCR (25 cycles) a DNA product of the expected size (~1.9 kb) was obtained, and when sequenced proved to be identical to the gene encoding the product YqiE. The PCR products from both reactions were treated with Taq DNA polymerase (GibcoBRL) at 72°C for 10 min to synthesise blunt ended fragments. The fragments were then cloned into the EcoRV site of the pBluescript vector (Stratagene) using T4 DNA ligase (Fermentas) (Fig. 2).

Substitute the following paragraph for the paragraph that begins on line 32, page 20 of the specification as filed.

The amino acid sequence of the DXPS proteins of Synechocystis sp. 6803 (SEQ ID NO: 1) and B. subtilis (SEQ ID NO: 2) exhibited significant similarity to each other over their entire length (47% identities) and to *E. coli* DXPS (SEQ ID NO: 3) (B. subtilis (44% identities) and Synechocystis sp. 6803 (46% identities)) (Fig. 3). All three polypeptides share two conserved domains; one thought to be involved in thiamin binding [30] and a histidine residue postulated to participate in proton transfer [31], both of which are detailed in Fig. 3. The existence of thiamin-

binding domain in each of the polypeptides explains the cofactor requirement of thiamin for DXPS activity [12]. The high degree of polypeptide sequence identity, particularly the distribution of conserved domains, in all three indicates that they all encode DXPS or a closely related gene product.

Substitute the following paragraph for the paragraph that begins on line 34, page 27 of the specification as filed.

Forward: 5'-GCG CCG CTA TTT ACT CGA-3' (SEQ ID NO: 11)

Substitute the following paragraph for the paragraph that begins on line 1, page 28 of the specification as filed.

Reverse: 5'-TTT CTC TGG CGT GCC GCC-3' (SEQ ID NO: 12)

A marked up copy of the specification amendments is attached as Appendix A to facilitate the Examiner's review.

Please substitute the enclosed Sequence Listing for the presently filed Sequence Listing.

In the Claims

Please amend the claims as follows:

5.(amended) A method according to claim 3 wherein said vector comprises one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a desired isoprenoid.

6.(amended) A method according to claim 3 wherein said plant or plant cell is transformed with a further vector comprising one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a desired isoprenoid.

7.(amended) A method according to claim 3 wherein said vector comprising said nucleic acid sequence(s) encoding said DXPS and/or said polypeptide(s) capable of producing said isoprenoid further comprises a nucleic acid sequence of either a tissue specific promoter and/or encoding a plastid transit peptide.

8.(amended) A method according to claim 5 wherein said desired isoprenoid is one conferring a nutritional benefit.

16.(amended) A method according to claim 14, wherein said cell is any of a bacterial, yeast or algal cell.

18.(amended) A method according to claim 14 wherein said organism is a plant.

19.(amended) A method according to claim 14 wherein said cell is any of a bacterial, yeast or algal cell.

20.(amended) A method according to claim 14 wherein said bacterial cell is *E. coli*.

27.(amended) A transgenic cell, tissue or organism according to claim 24, wherein said organism is a plant.

29.(amended) Progeny of the organism according to claim 24 having increased isoprenoid activity.

30.(amended) A transformed plant comprising a transgene capable of expressing DXPS from *E. coli* having the sequence according to SEQ ID NO: 3 and which plant comprises a higher level of isoprenoid than an untransformed plant.

31.(amended) A transformed plant according to claim 30 comprising any of constructs pVB6_TSEC_LML (SEQ ID NO: 6) or pVB6_35S_TSEC-LML (SEQ ID NO: 5).

32.(amended) A transformed plant according to claim 30 wherein said plant is a tomato plant.

A marked up copy of the amended claims is attached as Appendix B to facilitate the Examiner's review.

Remarks

Applicants have amended the specification to provide priority application information and information regarding the publication in English under PCT Article 21(2) of the PCT application of which the above-identified application is a U.S. national stage application. The specification also has been amended to insert sequence identifiers. A substitute Sequence Listing is filed herewith.

Applicants have amended the claims to eliminate certain dependencies and to add sequence identifiers. No new matter has been added.

Respectfully submitted,

John R. Van Amsterdam
Reg. No. 40,212
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2211
Tel: (617) 720-3500

Attorney's Docket No. B0192/7031
Dated: 27 July 2001

Appendix A

Added Section

Related Applications

This application is a national stage filing under 35 U.S.C. § 371 of PCT International application PCT/GB00/00263, and filed 28 January 2000, which was published under PCT Article 21(2) in English.

Foreign priority benefits are claimed under 35 U.S.C. § 119(a)-(d) of Great Britain application number GB 9901902.8, filed 28 January 1999.

Amended Paragraphs of the Specification

Substitute the following paragraph for the paragraph that begins on line 33, page 13 of the specification as filed.

Figure 3: is an illustration of an amino acid sequence alignment of *DXP* synthases used in the present invention, Synechocystis sp. 6803 (S.s) (GenBank D90903) (SEQ ID NO: 1), B. subtilis (B.s) (GenBank D84432) (SEQ ID NO: 2) and *E. coli* (E.c) (GenBank AF035440) (SEQ ID NO: 3). The consensus line (consen) shows residues conserved in all three sequences (upper case letters) or residues which are identical in two sequences and replaced by an equivalent amino acid in the third sequence (+). The conserved histidine domain putatively involved in proton transfer is over lined and numbered 1. The second over lined domain (2) denotes the consensus thiamin pyrophosphate (TPP)-binding motif.

Substitute the following paragraph for the paragraph that begins on line 35, page 14 of the specification as filed.

Figure 6: is a diagrammatic illustration of vector pVB6_TSEC_LML (SEQ ID NO: 6).

Substitute the following paragraph for the paragraph that begins on line 3, page 15 of the specification as filed.

Figure 7: is a diagrammatic representation of plasmid pVB6_35S_TSEC_LML (SEQ ID NO: 5).

Substitute the following paragraph for the paragraph that begins on line 6, page 15 of the specification as filed.

Figure 8: is an illustration of the amino acid sequence of *E. coli* DXPS (SEQ ID NO: 3).

Substitute the following paragraph for the paragraph that begins on line 9, page 15 of the specification as filed.

Figure 9: is an illustration of the transit peptide used in tomato plants (SEQ ID NO: 4).

Substitute the following paragraph for the paragraph that begins on line 15, page 16 of the specification as filed.

Based on the nucleotide sequence of ORF s111945 from the genome database for Synechocystis sp. PCC 6803 [23], primers were designed to clone the putative dxps gene by polymerase chain reaction (PCR). The forward primer 5'-GTCCCAATCCACCATGCACATCAG-3' (SEQ ID NO: 7) overlaps the beginning of the coding sequence. The reverse primer 5'-CCCTCGACAAATGCAAAATGTATC-3' (SEQ ID NO: 8) lies outside the stop codon of the gene. A PCR (25 cycles) using Pfu DNA polymerase (Stratagene) produced a DNA fragment of the expected size (~1.9 kb). Subsequent sequencing of the fragment confirmed the product to be the ORF s111945. The B. subtilis dxps gene was also cloned by PCR using primers designed to amplify the gene encoding the product YqiE, identified in the Bacillus subtilis genome database [24]. The forward primer 5'-GATCCGCTATGGATCTTTTATC-3' (SEQ ID NO: 9) contains a modified base substitution at the predicted start codon (underlined) for improved expression in *E. coli*. The reverse primer 5'-ATCTAATCGTTCTTTCTTTGAC-3' (SEQ ID NO: 10) lies outside

the stop codon of the dxps gene. After PCR (25 cycles) a DNA product of the expected size (~1.9 kb) was obtained, and when sequenced proved to be identical to the gene encoding the product YqiE. The PCR products from both reactions were treated with Taq DNA polymerase (GibcoBRL) at 72°C for 10 min to synthesise blunt ended fragments. The fragments were then cloned into the EcoRV site of the pBluescript vector (Stratagene) using T4 DNA ligase (Fermentas) (Fig. 2).

Substitute the following paragraph for the paragraph that begins on line 32, page 20 of the specification as filed.

The amino acid sequence of the DXPS proteins of Synechocystis sp. 6803 (SEQ ID NO: 1) and B. subtilis (SEQ ID NO: 2) exhibited significant similarity to each other over their entire length (47% identities) and to *E. coli* DXPS (SEQ ID NO: 3) (B. subtilis (44% identities) and Synechocystis sp. 6803 (46% identities)) (Fig. 3). All three polypeptides share two conserved domains; one thought to be involved in thiamin binding [30] and a histidine residue postulated to participate in proton transfer [31], both of which are detailed in Fig. 3. The existence of thiamin-binding domain in each of the polypeptides explains the cofactor requirement of thiamin for DXPS activity [12]. The high degree of polypeptide sequence identity, particularly the distribution of conserved domains, in all three indicates that they all encode DXPS or a closely related gene product.

Substitute the following paragraph for the paragraph that begins on line 34, page 27 of the specification as filed.

Forward: 5'-GCG CCG CTA TTT ACT CGA-3' (SEQ ID NO: 11)

Substitute the following paragraph for the paragraph that begins on line 1, page 28 of the specification as filed.

Reverse: 5'-TTT CTC TGG CGT GCC GCC-3' (SEQ ID NO: 12)

Appendix B

5. A method according to claim 3 [or 4] wherein said vector comprises one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a desired isoprenoid.

6. A method according to claim 3 [or 4] wherein said plant or plant cell is transformed with a further vector comprising one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a desired isoprenoid.

7. A method according to [any of] claim[s] 3 [to 6] wherein said vector comprising said nucleic acid sequence(s) encoding said DXPS and/or said polypeptide(s) capable of producing said isoprenoid further comprises a nucleic acid sequence of either a tissue specific promoter and/or encoding a plastid transit peptide.

8. A method according to [any of] claim[s] 5 [to 7] wherein said desired isoprenoid is one conferring a nutritional benefit.

16. A method according to claim 14 [or 15], wherein said cell is any of a bacterial, yeast or algal cell.

18. A method according to claim 14 [or 15] wherein said organism is a plant.

19. A method according to [any of] claim[s] 14 [to 18] wherein said cell is any of a bacterial, yeast or algal cell.

20. A method according to [any of] claim[s] 14 [to 19] wherein said bacterial cell is *E. coli*.

27. A transgenic cell, tissue or organism according to [any of] claim[s] 24 [to 26], wherein said organism is a plant.

29. Progeny of the organism according to [any of] claim[s] 24 [to 28] having increased isoprenoid activity.

30. A transformed plant comprising a transgene capable of expressing DXPS from *E. coli* having the sequence according to [Figure 8] SEQ ID NO: 3 and which plant comprises a higher level of isoprenoid than an untransformed plant.

31. A transformed plant according to claim 30 comprising any of constructs pVB6_TSEC_LML (SEQ ID NO: 6) or pVB6_35S_TSEC-LML (SEQ ID NO: 5) [illustrated in Figures 6 and 7].

32. A transformed plant according to claim 30 [or 31] wherein said plant is a tomato plant.

SEQUENCE LISTING

<110> Bramley, Peter Michael
Harker, Mark

<120> Manipulating Isoprenoid Expression

<130> B0192/7031

<140> 09/890,229
<141> 2000-01-28

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35 40 45

Val Glu Leu Thr Val Ala Leu Tyr Ser Thr Leu Asp Leu Asp Lys Asp
50 55 60

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65 70 75 80

Thr Gly Arg Tyr His Asp Phe His Thr Leu Arg Gln Lys Asp Gly Val
85 90 95

Ala Gly Tyr Leu Lys Arg Ser Glu Ser Arg Phe Asp His Phe Gly Ala
100 105 110

Gly His Ala Ser Thr Ser Ile Ser Ala Gly Leu Gly Met Ala Leu Ala
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Arg Asp Ala Lys Gly Glu Asp Phe Lys Val Val Ser Ile Ile Gly Asp
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Ser Ile Ser Pro Asn Val Gly Ala Ile Ser Arg Tyr Leu Asn Lys Val

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Arg Val Lys Glu Gly Met Lys Arg Leu Val Val Pro Lys Val Gly Ala
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Val Ile Glu Glu Leu Gly Phe Lys Tyr Phe Gly Pro Ile Asp Gly His
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Ser Leu Gln Glu Leu Ile Asp Thr Phe Lys Gln Ala Glu Lys Val Pro
            260                 265                 270

Gly Pro Val Phe Val His Val Ser Thr Thr Lys Gly Lys Gly Tyr Asp
        275                 280                 285

Leu Ala Glu Lys Asp Gln Val Gly Tyr His Ala Gln Ser Pro Phe Asn
    290                 295                 300

Leu Ser Thr Gly Lys Ala Tyr Pro Ser Ser Lys Pro Lys Pro Pro Ser
305                 310                 315                 320

Tyr Ser Lys Val Phe Ala His Thr Leu Thr Thr Leu Ala Lys Glu Asn
                325                 330                 335

Pro Asn Ile Val Gly Ile Thr Ala Ala Met Ala Thr Gly Thr Gly Leu
            340                 345                 350

Asp Lys Leu Gln Ala Lys Leu Pro Lys Gln Tyr Val Asp Val Gly Ile
        355                 360                 365

Ala Glu Gln His Ala Val Thr Leu Ala Ala Gly Met Ala Cys Glu Gly
    370                 375                 380

Ile Arg Pro Val Val Ala Ile Tyr Ser Thr Phe Leu Gln Arg Gly Tyr
385                 390                 395                 400

Asp Gln Ile Ile His Asp Val Cys Ile Gln Lys Leu Pro Val Phe Phe
                405                 410                 415

Cys Leu Asp Arg Ala Gly Ile Val Gly Ala Asp Gly Pro Thr His Gln
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Gly Met Tyr Asp Ile Ala Tyr Leu Arg Cys Ile Pro Asn Leu Val Leu
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Met Ala Pro Lys Asp Glu Ala Glu Leu Gln Gln Met Leu Val Thr Gly
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Val Asn Tyr Thr Gly Gly Ala Ile Ala Met Arg Tyr Pro Arg Gly Asn
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        595                 600                 605

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Thr Ser Leu Ser Ala Ser Gly Gly His Ile Gly Pro Asn Leu Gly Val
        35                  40                  45

Val Glu Leu Thr Val Ala Leu His Lys Glu Phe Asn Ser Pro Lys Asp
    50                  55                  60

Lys Phe Leu Trp Asp Val Gly His Gln Ser Tyr Val His Lys Leu Leu
65                  70                  75                  80

Thr Gly Arg Gly Lys Glu Phe Ala Thr Leu Arg Gln Tyr Lys Gly Leu
                85                  90                  95

Cys Gly Phe Pro Lys Arg Ser Glu Ser Glu His Asp Val Trp Glu Thr
            100                 105                 110

Gly His Ser Ser Thr Ser Leu Ser Gly Ala Met Gly Met Ala Ala Ala
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Arg Asp Ile Lys Gly Thr Asp Glu Tyr Ile Ile Pro Ile Ile Gly Asp
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Asp Glu Lys Lys Asp Met Ile Val Ile Leu Asn Asp Asn Glu Met Ser
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Ile Ala Pro Asn Val Gly Ala Ile His Ser Met Leu Gly Arg Leu Arg
            180                 185                 190

Thr Ala Gly Lys Tyr Gln Trp Val Lys Asp Glu Leu Glu Tyr Leu Phe
        195                 200                 205

Lys Lys Ile Pro Ala Val Gly Gly Lys Leu Ala Ala Thr Ala Glu Arg
    210                 215                 220

Val Lys Asp Ser Leu Lys Tyr Met Leu Val Ser Gly Met Phe Phe Glu
225                 230                 235                 240

Glu Leu Gly Phe Thr Tyr Leu Gly Pro Val Asp Gly His Ser Tyr His
                245                 250                 255

Glu Leu Ile Glu Asn Leu Gln Tyr Ala Lys Lys Thr Lys Gly Pro Val
            260                 265                 270

Leu Leu His Val Ile Thr Lys Lys Gly Lys Gly Tyr Lys Pro Ala Glu
        275                 280                 285

Thr Asp Thr Ile Gly Thr Trp His Gly Thr Gly Pro Tyr Lys Ile Asn
    290                 295                 300

Thr Gly Asp Phe Val Lys Pro Lys Ala Ala Ala Pro Ser Trp Ser Gly
305                 310                 315                 320

Leu Val Ser Gly Thr Val Gln Arg Met Ala Arg Glu Asp Gly Arg Ile
                325                 330                 335

Val Ala Ile Thr Pro Ala Met Pro Val Gly Ser Lys Leu Glu Gly Phe
            340                 345                 350

Ala Lys Glu Phe Pro Asp Arg Met Phe Asp Val Gly Ile Ala Glu Gln
        355                 360                 365

His Ala Ala Thr Met Ala Ala Ala Met Ala Met Gln Gly Met Lys Pro
    370                 375                 380

Phe Leu Ala Ile Tyr Ser Thr Phe Leu Gln Arg Ala Tyr Asp Gln Val
385                 390                 395                 400

Val His Asp Ile Cys Arg Gln Asn Ala Asn Val Phe Ile Gly Ile Asp
                405                 410                 415

Arg Ala Gly Leu Val Gly Ala Asp Gly Glu Thr His Gln Gly Val Phe
            420                 425                 430

Asp Ile Ala Phe Met Arg His Ile Pro Asn Met Val Leu Met Met Pro
        435                 440                 445

Lys Asp Glu Asn Glu Gly Gln His Met Val His Thr Ala Leu Ser Tyr
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Attorney's Docket No: B0192/7031

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Peter BRAMLEY et al.
International Application No.	:	PCT/GB00/00263
International Filing Date	:	28 January 2000 (28.01.00)
Earliest Priority Date	:	28 January 1999 (28.01.99)
Title	:	MANIPULATING ISOPRENOID EXPRESSION

Commissioner for Patents
Box PCT
Washington, D.C. 20231

STATEMENT UNDER 37 C.F.R. §1.821(f)

Sir:

This statement is made pursuant to 37 CFR 1.821(f). Applicants enclose herewith an original written copy of the Sequence Listing and a computer readable diskette. Applicants' attorney states that the information recorded in the computer readable form is identical to the written Sequence Listing and that the Sequence Listing contains no new matter.

Respectfully submitted,

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Docket No. B0192/7031
Dated: July 27, 2001
xNDD

MANIPULATING ISOPRENOID EXPRESSION

The present invention is concerned with manipulating or altering isoprenoid expression in a cell or organism which biosynthesises isopentenyl diphosphate (IPP), which is the universal precursor of all isoprenoids in nature, via a mevalonate independent pathway.

Isoprenoids constitute the largest class of natural products occurring in nature, with over 29,000 individual compounds identified to date [1]. Chemically, they are extremely diverse in their structure and complexity. The fundamental biological functions performed by isoprenoids ensure they are essential for the normal growth and developmental processes in all living organisms. These include functioning as eukaryotic membrane stabilisers (sterols), plant hormones (gibberellins and abscisic acid), providing pigments for photosynthesis (carotenoids and phytol side chain of chlorophyll), and as carriers for electron transport (menaquinone, plastoquinone and ubiquinone).

All isoprenoids are synthesised via a common metabolic precursor, isopentenyl diphosphate (IPP; C_5). Until recently, the biosynthesis of IPP was generally assumed to proceed exclusively from acetyl-CoA via the classical mevalonate pathway (Fig. 1) [2]. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) catalyses the conversion of hydroxymethylglutaryl-CoA to mevalonate, a key reaction of the mevalonate-dependant IPP biosynthetic pathway. Recent studies have demonstrated that mevalonate is not the biosynthetic precursor of IPP in all living organisms

[3,4]. The existence of an alternative, mevalonate-independent pathway for IPP formation was identified initially in several species of eubacteria [4,5] and a green alga [6]. The pathway was subsequently shown to be operational in the plastids of higher plants [7-10]. The first reaction in the non-mevalonate pathway is the transketolase-type condensation reaction of pyruvate and D-glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (DXP) (Fig. 1). This reaction is catalysed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase. The second reaction in the pathway is the conversion of DXP to 2-C-methyl-D-erythritol-4-phosphate (MEP). The reactions which convert MEP to IPP have yet to be characterised.

The cloning and characterisation of the DXP synthase (*dxps*) gene has been described for a number of organisms including Esherichia coli [11,12] and higher plants [13-15]. The *CLA1* gene product from *Arabidopsis thaliana* associated with chloroplast development [16], for example, has been shown to exhibit *DXPS* activity [11]. Recently, a gene responsible for the reduction of DXP to 2-C-methyl-D-erythritol-4-phosphate, the proposed next step in the non-mevalonate pathway has been cloned from *E. coli* [17].

The present inventors have surprisingly found that the first reaction in the mevalonate-independent IPP biosynthetic pathway is highly influential in controlling the levels of isoprenoids which can be formed in a cell or organism within which the mevalonate independent IPP biosynthetic pathway is present. The enzyme DXPS or functional equivalents

thereof, has been identified by the present inventors as a rate-limiting step in isoprenoid biosynthesis and that DXPS activity plays an important role in channelling the carbon resources of the cell into the isoprenoid biosynthetic pathway.

Therefore, according to a first aspect of the present invention there is provided a method of manipulating isoprenoid expression in a cell possessing a mevalonate independent isopentenyl diphosphate synthesising pathway, which method comprises altering the activity of the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), or a functional equivalent thereof. Thus, advantageously, the rate-limiting effect conferred by DXPS activity on the IPP biosynthetic pathway can be utilised to manipulate the resultant levels of isoprenoids in a cell by altering the activity or expression of DXPS.

Preferably, the levels of isoprenoids in a cell can be enhanced by increasing the activity or expression of the DXPS. Likewise reduced levels of isoprenoids can be achieved by reducing or inhibiting activity or expression of DXPS in a cell or organism. Increasing the DXPS activity may be achieved by, for example, transforming the cell which may itself be part of a cell line or an organism, with an expression vector comprising a nucleic acid molecule encoding DXPS, which may advantageously be operably linked to a reporter molecule, such as used in the GUS assay which is known in the art. Preferably, the vector comprises any of the vectors designated pBSDXPS or pSYDXPS, illustrated in Figure 2.

An alternative method for altering expression may comprise utilising a technique known as Enforced Evolution, or DNA Shuffling see Patten *et al.* Current Opinion in Biotechnology, 1997, Vol. 8, No. 6, pp 724-733, Crameri *et al.*, Nature 1998, Vol. 391, No. 6664, pp 288-291 and Harayama S, Trends in Biotechnology, 1998, Vol. 16, No. 2, pp 76-82. According to this method improvements in enzyme activity can be achieved by reassembling DNA segments into a full length gene by homologous or site specific combination. Before the assembly, the segments are often subjected to random mutagenesis by error prone PCR, random nucleotide insertion, or other such methods. The genes can be expressed in suitable microbial hosts leading to the production of functional polypeptides, such as DXPS.

The nucleic acid encoding the DXPS may be endogenous to the cell or organism into which it will be transformed or, alternatively, may be exogenous. In one embodiment of the invention, the method may also comprise transforming the cell or organism with a vector comprising one or more nucleic acid sequences suitable for producing a desired isoprenoid. This aspect of the invention is particularly advantageous because it allows isoprenoids to be produced in a cell or organism independent of the source of the isoprenoid which may be derived from cells or organisms which do not possess the mevalonate independent IPP biosynthesising pathway. Similarly, enhanced levels of an isoprenoid can be produced in cells or organisms having the mevalonate independent IPP biosynthetic pathway.

Therefore, in the example where the cell is *E. coli* it is possible to engineer production of an isoprenoid which is exogenous to the *E. coli* bacterium, which isoprenoid may be, for example, any of the carotenoids of plants, such as, lycopene or even an isoprenoid of human origin.

Carotenoids are yellow-orange-red lipid based pigments found in nature. They have been found to be useful in a variety of applications, for example, as supplements, and particularly vitamin supplements, as vegetable oil based food products and food ingredients, as feed additives in animal feeds and as colorants. Phytoene has been found to be useful in treating skin disorders whilst lycopene and α and β carotene consumption have been implicated as having preventative effects against certain kinds of cancers. Therefore, it is a highly advantageous aspect of the invention that increased production of such compounds can be achieved and which compounds can confer considerable health care benefits. Once the desired carotenoid or other isoprenoid has been produced in *E. coli*, or other suitable organism as defined above, it can be isolated using standard bicengineering techniques.

Increases in concentrations of any desired isoprenoid may be achieved, in a cell or alternatively an organism which possesses the IPP biosynthetic mevalonate independent pathway. For example, crops can be engineered using the method of the invention to produce increased levels of an isoprenoid which confers nutritional benefits to humans following consumption of the plant, such as, for example, vitamin E and lycopene.

Therefore, there is also provided by a further aspect of the invention a cell or organism having a mevalonate independent IPP biosynthetic pathway and which has been transformed or transfected with an expression vector comprising a nucleic acid molecule encoding DXPS or a functional equivalent or bioprecursor thereof. As described above, the vector may also include one or more further nucleic acid sequences suitable for producing a desired isoprenoid, or alternatively the one or more nucleic acid sequences may be included in a separate vector, operably linked to suitable expression control sequences. In a particularly preferred embodiment the cell or organism comprises a plant.

An expression vector according to the invention includes a vector having a nucleic acid sequence operably linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of said DNA fragments. The term "operably linked" refers to a juxta position wherein the components described are in a relationship permitting them to function in their intended manner. Such vectors may be transformed into a suitable host cell or organism to produce a desired protein, such as DXPS or an isoprenoid according to the method of the invention. Thus, in a further aspect, the invention provides a process for producing a desired isoprenoid which comprises cultivating a host cell, transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of DXPS or a functional equivalent thereof or suitable polypeptides for producing a desired isoprenoid and optionally recovering the expressed polypeptides.

The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of said nucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable markers, such as, for example, ampicillin resistance.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and for transcription initiation in the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art.

By combining the nucleic acid sequences encoding said DXPS and optionally the one or more sequences suitable for producing an isoprenoid with tissue specific promoters, enhanced isoprenoid levels in specified tissues of plants can be achieved. For example, by utilising a seed specific promoter or other transcriptional initiation region, elevated levels of carotenoids in seeds may be achieved. The seed can then be harvested and which provides a reservoir for the isoprenoid or carotenoid of interest.

Generally, the nucleic acid molecule encoding said DXPS which is included in the vector used in

accordance with the method of the invention, will be transformed into a plant cell so that the DXPS molecule is directed to the plastids of the plant. Accordingly, where the vector is not inserted directly into the plastid of the plant, the vector will further comprise a nucleic acid sequence operably linked to said DXPS or said one or more isoprenoid producing nucleic acid sequences and which further sequence will encode a transit peptide to direct expression of the DXPS or isoprenoid into the plastid. Native or heterologous transit peptides may be utilised in this embodiment of the invention.

As aforesaid, the mevalonate independent IPP biosynthetic pathway is not present in any higher animals, particularly humans. Therefore, the inhibition of the reaction catalysed by DXPS provides a unique target site to selectively inhibit or alleviate bacterial associated infections by altering the expression level of or inhibiting function or activity of DXPS.

One method of inhibiting or preventing expression of DXPS utilises antisense technology. Antisense technology can be used to control gene expression through helix formation of antisense DNA or RNA, both of which methods are based on polynucleotide binding to DNA or RNA. For example, the 5'-coding region of a native DNA sequence coding for DXPS according to the invention may be used to design an antisense RNA nucleotide of from 10 to 50 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee *et al*, Nucl. Acids. Res., 6:3073 (1979); Cooney *et al.*, Science, 241:456 (1988);

and Derman *et al.*, Science 251:1360 (1991), in which case expression of the antisense RNA oligonucleotide allows hybridisation to the mRNA *in vivo* and blocks translation of an mRNA molecule into DXPS.

Alternatively, compounds can be screened for their ability to inhibit the catalytic activity or expression of DXPS in the mevalonate - independent IPP biosynthetic pathway. According to a further aspect of the invention, therefore, there is also provided a method of identifying a compound which modulates isoprenoid production or expression which method comprises contacting said compound to be tested with a molecule from the mevalonate independent IPP biosynthetic pathway and which molecule undergoes a reaction in the presence of an appropriate reactant catalysed by DXPS, in the presence of DXPS and monitoring the level of product produced when compared to the same reaction in the absence of the compound to be tested. Preferably, the molecules which are reacted are pyruvate and glyceraldehyde-3-phosphate, and which undergo a condensation reaction in the presence of DXPS, to yield 1-deoxy-D-xylulose-5-phosphate (DXP) as illustrated in Figure 1.

Any compounds identified as preventing expression or activity of the DXPS enzyme according to the invention may advantageously be particularly useful as selective toxicity agents to destroy, for example, bacterial or plant cells which possess the mevalonate independent IPP biosynthetic pathway. These compounds therefore can be particularly useful as medicaments or herbicides, or alternatively in the preparation of a medicament for treating bacterial associated diseases.

A further aspect of the invention therefore also comprises a pharmaceutical composition comprising a compound identified as an inhibitor of expression or activity of DXPS or a functional equivalent or bioprecursor thereof, together with a pharmaceutically acceptable carrier, diluent or excipient thereof. Also provided by the invention is a herbicidal composition comprising said compound identified as an inhibitor of expression or activity of DXPS function.

An even further aspect of the invention comprises a transgenic cell, tissue or organism having a mevalonate independent IPP biosynthetic pathway, which comprises a transgene capable of expressing at least one additional DXPS molecule according to the invention. The transgenic cell, tissue or organism may also comprise a transgene having one or more nucleic acid sequences capable of producing a desired isoprenoid. Preferably, the transgenic cell comprises a plant and even more preferably tomato plants.

The term "transgene capable of expression" as used herein means a suitable nucleic acid sequence(s) which leads to expression of DXPS or proteins having the same function and/or activity and/or encoding proteins capable of producing a desired isoprenoid. The transgene, may include, for example, isolated genomic nucleic acid or synthetic nucleic acid, including DNA integrated into the genome. Preferably, the transgene comprises the nucleic acid sequence(s) encoding the DXPS enzyme or said isoprenoid as described herein, or a functional fragment of said nucleic acid. A functional fragment of said nucleic acid should be taken to mean a fragment of the gene comprising said nucleic acid(s) coding for the DXPS enzyme or said

isoprenoid or a functional equivalent, derivative or a non-functional derivative such as a dominant negative mutant, or bioprecursor thereof. For example, it would be readily apparent to persons skilled in the art that nucleotide substitutions or deletions may be made using routine techniques, which do not affect the protein sequence and subsequent functioning of the DXPS enzyme and/or isoprenoid producing proteins encoded by said nucleic acid(s).

The DXPS enzyme expressed or the isoprenoid produced by said transgenic cell, tissue or organism or a functional equivalent or bioprecursor of said protein also forms part of the present invention.

The recombinant DNA molecules or vectors of the invention can be introduced into a plant cell in a number of recognised ways in the art and it will be appreciated that the choice of method used might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) BioTechnieques 4:320-334), electroporation (Riggs *et al.* (1986) Proc. Natl. Acad. Sci. USA 83:5602-5606, Agrobacterium mediated transformation (Hinchee et al. (1988) Biotechnology 6:915-921) and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al.* (1988) Biotechnology 6:923-926).

Alternatively, in the case of an organism, such as a plant, a plastid can be transformed directly. Stable transformation of chloroplasts has been reported in higher plants, see, for example, SVAB *et al.* (1990) Proc. Natl. Acad. Sci. USA 87:8526-8530; SVAB & Maliga

(1993) Proc. Natl. Acad. Sci. USA 90:913-917; Staub & Maliga (1993) Embo J. 12:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. In such methods, plastid gene expression can be accomplished by use of a plastid gene promoter or by trans-activation of a silent plastid-borne transgene positioned for expression from a selective promoter sequence such as that recognised by T7 RNA polymerase. The silent plastid gene is activated by expression of the specific RNA polymerase from a nuclear expression construct and targeting of the polymerase to the plastid by use of a transit peptide. Tissue-specific expression may be obtained in such a method by use of a nuclear-encoded and plastid-directed specific RNA polymerase expressed from a suitable plant tissue specific promoter. Such a system has been reported in McBride *et al.* (1994) Proc. Natl. Acad. Sci., USA 91:7301-7305.

The cells which have been transformed may be grown into plants in accordance with conventional methods known in the art. See, for example, McCormick *et al.*, Plant Cell Reports (1986), 5:81-84. These plants may then be grown, and either pollinated with the same transformed strainer or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

A host cell of any plant variety may be employed.

Plant species which provide seeds of interest are particularly useful. For the most part, plants will be chosen where the seed is produced in high amounts, a seed-specific product of interest is involved, or the seed or a seed part is edible. Seeds of interest include the oil seeds, such as oilseed Brassica seeds, cotton seeds, soybean, safflower, sunflower, coconut, palm, and the like; grain seeds, e.g. wheat, barley, oats amaranth, flax, rye, triticale, rice, corn, etc.; other edible seeds or seeds with edible parts including pumpkin, squash, sesame, poppy, grape, mung beans, peanut peas, beans, radish, alfalfa, cocoa, coffee, tree nuts such as walnuts, almonds, pecans, chick-peas etc.

The invention may be more clearly understood from the following exemplary embodiment described with reference to the accompanying drawings wherein:

Figure 1: is an illustration of the mevalonate-dependant (A) and independent (B) pathways for IPP biosynthesis. Proposed reactions for the biosynthesis of 1-deoxy-D-xylulose-5-phosphate from pyruvate and glyceraldehyde-3-phosphate, catalysed by *DXPS* is as shown inside the box.

Figure 2: is an illustration of structure of plasmids pBSDXPS and pSYDXPS.

Figure 3: is an illustration of an amino acid sequence alignment of *DXP* synthases used in the present invention,

Synechocystis sp. 6803 (S.s) (GenBank D90903), B. subtilis (B.s) (GenBank D84432) and *E. coli* (E.c) (GenBank AF035440). The consensus line (consen) shows residues conserved in all three sequences (upper case letters) or residues which are identical in two sequences and replaced by an equivalent amino acid in the third sequence (+). The conserved histidine domain putatively involved in proton transfer is over lined and numbered 1. The second over lined domain (2) denotes the consensus thiamin pyrophosphate (TPP)-binding motif.

Figure 4: is a graphic representation of lycopene accumulation in recombinant *E. coli* cultures expressing vector only (□), B. subtilis DXPS (•) and Synechocystis sp. 6803 DXPS (△). (Data are means ± S.E.M. from three independent determinations.)

Figure 5: is an illustration of lycopene (open columns) and UQ-8 (shaded columns) content of *E. coli* control cultures (vector only) or expressing exogenous B. subtilis dxps (B. subtilis), Synechocystis sp. 6803 dxps (sp. 6803) or A. thaliana hmgr1 (HMGR1) genes. (Data are means (S.E.M. from three independent determinations.)

Figure 6: is a diagrammatic illustration of

vector pVB6_TSEC_LML.

Figure 7: is a diagrammatic representation of plasmid pVB6_35S_TSEC-LML.

Figure 8: is an illustration of the amino acid sequence of *E.coli* DXPS.

Figure 9: is an illustration of the transit peptide used in tomato plants.

EXAMPLE 1

Materials and methods

Bacterial strains, plasmids, and culture conditions.

E. coli strain XL1-Blue (Stratagene) was used for gene cloning and expression of plasmids. *E. coli* was grown in Luria Broth media [18] at 37°C on a rotary shaker at 250 rpm (unless otherwise stated). Ampicillin (100 μg/ml), chloramphenicol (50 μg/ml) and 1.0 mM isopropyl-b--D-thiogalactoside (IPTG) (all purchased from Sigma) were added as required. Plasmid pBluescript (Stratagene) was used as a vector for both cloning and expression studies. Synechocystis sp. PCC 6803 was obtained from the Institute Pasteur (Paris) and grown in BG11 medium [19] supplemented with 0.5% glucose at 30°C and 2,000 lux. Bacillus subtilis strain PY79 DNA was a kind gift from P. Wakeley (Royal Holloway, University of London). The construction of plasmid pACCRT-EIB, which expresses the E. uredovora crtE, crtB and crtI genes necessary for lycopene biosynthesis in *E. coli* cells into which it has been

introduced, has been described previously [20]. The plasmid used for the expression of HMGR1 cloned into pBluescript (pHMGR1) has also been described elsewhere [21].

Recombinant DNA techniques

All recombinant DNA techniques were performed by standard methods [22] or according to suppliers instructions. Genomic DNA was extracted from all organisms using the Qiagen Genomic-tip 20/G kit.

Cloning of dxps genes

Based on the nucleotide sequence of ORF sll1945 from the genome database for Synechocystis sp. PCC 6803 [23], primers were designed to clone the putative dxps gene by polymerase chain reaction (PCR). The forward primer 5'-GTCCCAATCCACCATGCACATCAG-3' overlaps the beginning of the coding sequence. The reverse primer 5'-CCCTCGACAAATGCAAAATGTATC-3' lies outside the stop codon of the gene. A PCR (25 cycles) using Pfu DNA polymerase (Stratagene) produced a DNA fragment of the expected size (~1.9 kb). Subsequent sequencing of the fragment confirmed the product to be the ORF sll1945. The B. subtilis dxps gene was also cloned by PCR using primers designed to amplify the gene encoding the product YqiE, identified in the Bacillus subtilis genome database [24]. The forward primer 5'-GATCCGCTATGGATCTT TTATC-3' contains a modified base substitution at the predicted start codon (underlined) for improved expression in *E. coli*. The reverse primer 5'-ATCTAATCGTTCTTTCTTTGAC-3' lies outside the stop codon of the dxps gene. After PCR (25 cycles) a DNA product of the expected size (~1.9 kb) was

obtained, and when sequenced proved to be identical to the gene encoding the product YqiE. The PCR products from both reactions were treated with Taq DNA polymerase (GibcoBRL) at 72°C for 10 min to synthesise blunt ended fragments. The fragments were then cloned into the EcoRV site of the pBluescript vector (Stratagene) using T4 DNA ligase (Fermentas) (Fig. 2).

In vitro DXP synthase assay

E. coli XL1-blue cells, transformed with the appropriate plasmid, were grown at 37°C in Luria Broth medium with appropriate antibiotics to an $OD_{620\ nm}$ of 0.6, and induced by the addition of 1.0 mM IPTG at 28°C for two hours. Bacteria were harvested by centrifugation (6,000g for 10 min) and washed in buffer A (100 mM Tris-HCl (pH 7.5), 1 mM dithioreitol, 0.3 M sucrose). Cells were resuspended to their original volume in buffer B (100 mM Tris (pH 8.0), 1 mM dithioreitol, 0.1 mM phenylmethanesulphonyl fluoride, 1μ/ml pepstatin, 1μg/ml leupeptin, 1 mg/ml lysozyme). The cells were then incubated at 30°C for 15 min with gentle agitation, and then disrupted by brief sonication at 4°C. The supernatant was recovered and the protein concentration determined using the Bradford assay [25].

An aliquot of the supernatant (100 μl) was transferred to an Eppendorf tube along with 100 μl of assay buffer containing 100 mM Tris (pH 8.0), 3 mM ATP, 3 mM Mn^{2+}, 3 mM Mg^{2+}, 1 mM KF, 1 mM thiamine diphosphate, (0.1%) Tween 60, 0.6 Mm mDL-glyceraldehyde-3-phosphate, 30 μM [2-^{14}C]pyruvate (0.5 μCi). The mixture was incubated for 2 hours at

30°C with gentle agitation. The reaction was stopped by heating the mixture at 80°C for 3 min. After centrifugation at 13,000 g for 5 min, the supernatant was transferred to a clean tube and evaporated to dryness. The residue was resuspended in methanol (50 μl) and loaded onto a TLC plate (silica gel 60). Chromatograms were developed in *n*-propyl alcohol/ethyl acetate/H_2O (6:1:3 v/v/v).

Enzyme assays were performed with extracts of induced cells expressing either Synechocystis sp. PCC 6803 or B. subtilis DXPS, as opposed to control assays in which cells contained only the pBluescript vector without insert. TLC analysis of assays expressing one of the dxps clones exhibited a major band (R_f 0.14) assumed to be DXP which was not observed in the controls. Quantification of ^{14}C-labelled DXP was achieved by isolation of the reaction product on TLC. The DXP band was scraped off the plate, eluted from the silica using methanol and quantified by liquid-scintillation counting. Enzymatic dephosphorylation of the assay products resulted in the formation of 1-deoxy-D-xylulose (DX), when analysed on TLC (R_f 0.50). When non-radioactive pyruvate was used in the assay, the DXP (Rf 0.12 stained purple) and DX (R_f 0.47 stained blue) were identified by staining with p-anisaldehyde/sulphuric acid (3:1). The DXP co-chromatographed with authentic, chemically synthesised DXP which stained purple also. The reaction substrates pyruvate (R_f 0.36 stained yellow), DL-glyceraldehyde-3-phosphate (R_f 0.15 stained orange) and D-glyceraldehyde (R_f 0.74 stained orange) were also observable using this TLC system. In reactions where the assay products were dephosphorylated no DXP was observed on TLC only DX.

Quantification of lycopene and ubiquinone QB-8 in *E. coli*

Bacterial growth was determined from the $OD_{620\ nm}$. Dry cell weight was calculated from known volumes of culture harvested by centrifugation at 13,000 g for 5 min, washed once with water and recentrifuged. The cells were lyophilised overnight and the weight of the dried cell pellet determined. The lycopene content of the cells was determined by harvesting aliquots of *E. coli* cells by centrifugation at 13,000 g for 5 min and washing once in water followed by recentrifuging. The cells were resuspended in acetone (200 μl) and incubated at 68°C for 5 min in the dark. The samples were centrifuged again 13,000 g for 10 min and the acetone supernatant containing the lycopene was placed in a clean tube. The extract was evaporated to dryness under a stream of N2 and stored at -20°C in the dark. The lycopene content of the extracts was determined by visible light absorption spectra using a Beckman DU Series 7000 diode array spectrometer. Spectra were recorded in acetone using an $A^{1\%}_{1cm}$ of 3450 [26].

UQ-8 was extracted from cells based on the methods of Yoshida et al. [27]. Cells were collected by centrifugation, washed once with water and then lyophilised overnight. The dried pellet was extracted in *n*-propanol (3 ml) and of *n*-hexane (5 ml) containing 15 μg of UQ-10 as an internal standard, by disruption of the cells using a pestle and mortar. The solvent phase and that obtained by the second extraction from the aqueous phase n-hexane (3 ml) were combined and evaporated to dryness under N_2. The residue was resuspended in ethanol and analysed by reversed phase

HPLC as described previously [28]. Peaks were identified by comparing their elution profiles with standards for UQ-7, UQ-9 and UQ-10. A standard of UQ-8 was not available, and the UQ-8 peak was identified by its elution profile relative to those of the other standards [29].

Cloning of the dxps genes

The cloning of dxps and the characterisation of the gene product, DXPS, from *E. coli* has recently been reported by two research groups [11,12]. The gene product was shown to exhibit DXP synthase activity, which is considered as the first reaction of the mevalonate-independent pathway for IPP biosynthesis (Fig. 1) [5]. Based on the *E. coli* dxps nucleotide sequence homologs of the gene were identified in the eubacterial genomes of B. subtilis and Synechocystis sp. PCC 6803. The open reading frame sll1945 in the Synechocystis sp. 6803 genome was cloned by PCR, ligated into the vector pBluescript, and designated pSYDXPS (Fig. 2). The gene extends over 1920 bp and contains an open reading frame encoding a polypeptide of 640 amino acids, with a predicted molecular mass of 69 kDa. The dxps homolog in the B. subtilis genome was identified as the ORF encoding the product YqiE. It was cloned by PCR, and introduced into pBluescript to generate plasmid pBSDXPS (Fig. 2). The gene extends over 1899 bp and encodes a polypeptide of 633 amino acids with a predicted molecular mass of 70 kDa.

The amino acid sequence of the DXPS proteins of Synechocystis sp. 6803 and B. subtilis exhibited significant similarity to each other over their entire length (47% identities) and to the *E. coli* DXPS (B.

subtilis (44 % identities) and Synechocystis sp. 6803 (46 % identities)) (Fig. 3). All three polypeptides share two conserved domains; one thought to be involved in thiamin binding [30] and a histidine residue postulated to participate in proton transfer [31], both of which are detailed in Fig. 3. The existence of a thiamin-binding domain in each of the polypeptides explains the cofactor requirement of thiamin for DXPS activity [12]. The high degree of polypeptide sequence identity, particularly the distribution of conserved domains, in all three indicates that they all encode DXPS or a closely related gene product.

Quantification of lycopene and UQ-8 in *E. coli* transformants

Cells of *E. coli* transformed with pACCRT-EIB [20] are pigmented pink due to the accumulation of lycopene. *E. coli* cells engineered to produce lycopene, were transformed with either pBSDXPS, pSYDXPS, pHMGR, or pBluescript to act as a control, to monitor the effect on lycopene biosynthesis when exogenous DXPS was expressed in the cells. The *E. coli* were grown in 50 ml cultures at 30°C with induction by IPTG for 48 hours, by which time they had reached the stationary phase of growth. Figure 4 shows the accumulation of lycopene in the cultures during the 48 hour culture period. The graph clearly demonstrates that the *E. coli* cultures expressing exogenous dxps accumulated lycopene at a much greater rate than the control culture. The final lycopene content of the recombinant dxps strains was approximately double that of the control (Fig. 5). A similar increase was also obtained in *E. coli* cells engineered to produce the

this organism.

In vitro enzyme activity

The increased levels of carotenoids and UQ-8 in *E. coli* expressing exogenous DXPS were hypothesised to be due to increased DXPS enzymatic activity in the cells. This was confirmed by preparing cell homogenates from recombinant *E. coli* strains after induction with IPTG. Reaction products were measured over a two hour period, separated by TLC and quantified by liquid-scintillation counting. The major product obtained from the reaction co-chromatographed with chemically-synthesised DXP. This confirms DXP as the major reaction product in the assay. The putative DXPS function of B. subtilis ORF encoding the product YqiE and Synechocystis sp. 6803 ORF sll1945 has been established by these results. Table 1 shows the specific activity of DXPS in the recombinant *E. coli* strains. The results show that DXPS activity was increased in *E. coli* expressing endogenous dxps genes. This increase was greatest in homogenates containing the B. subtilis DXPS, where a 2.0 fold increase was observed compared to the controls. Homogenates containing the Synechocystis sp. 6803 DXPS exhibited a 1.8 fold increase compared to control reactions. Therefore, increased DXPS activity in *E. coli* appears to be responsible for the increased levels of carotenoids and UQ-8 observed in the transgenic strains. The relative increases in carotenoid levels between *E. coli* cultures expressing plasmids pSYNDXSP and pBSDXPS closely resemble the increases observed in the in vitro studies. This suggests that there is a direct relationship between DXPS activity and the carotenoid content of the cells. This is not the case

colourless carotenoid phytoene (data not shown). Alterations in the endogenous levels of isoprenoids were determined by measuring the ubiquinone content of the cells. In *E. coli*, the major quinones encountered are ubiquinone (UQ-8) and menaquinone (MK-8) [32]. Ubiquinone is a major component of the aerobic respiratory chain. It is estimated that there are approximately 50 molecules of ubiquinone for each of the oxidation complexes in *E. coli* [33]. By measuring an end product which is produced in relatively large quantities, it was conjectured that alterations in the rates of biosynthesis could be readily detected. The UQ-8 content of the recombinant dxps strains was 1.5 times greater than the controls (Fig. 5). Lycopene and UQ-8 levels were measured in *E. coli* transformed with hmgr1 from A. thaliana, to monitor if this caused any alterations in the isoprenoid content of the cells. Expression of the A. thaliana hmgr1 cDNA had no effect of the levels of lycopene nor UQ-8 in the cells (Fig. 5).

The results show that increased expression of DXPS leads to increased lycopene and UQ-8 levels in the recombinant *E. coli* cells. This indicates that increasing the rate of DXP synthesis, the initial reaction in the mevalonate-independent pathway for IPP biosynthesis, elevates isoprenoid production. In contrast, expression of hmgr1 had no effect on isoprenoid biosynthesis, suggesting that mevalonate dependent IPP biosynthesis has little or no role in IPP synthesis in *E. coli*. Similarity searches of the *E. coli* genome data base for proteins of the mevalonate-dependent IPP biosynthesis pathway failed to identify any possible homologs in the genome suggesting that this pathway is probably absent in

for UQ-8 where increases in the levels of UQ-8 are more restricted, which could be due to a rate-limiting reaction later in the UQ-8 biosynthesis pathway [34]. The results support the hypothesis that increased DXPS activity in *E. coli* results in increased levels of carotenoids and UQ-8. These data suggest that isoprenoid levels in *E. coli* can be increased by enhancing DXPS activity.

Transformation Protocols in Tomato Plants

Triparental mating

Liquid LB medium (5ml) containing rifampicin (100μg/ml) was inoculated with a single *Agrobacterium tumefaciens* colony picked from an LB/rif plate. It was then incubated in a 27°C shaking incubator (225-250rpm) for 48 hours in the dark. Single colonies of Helper strain *E.coli* HB101/pRK2013 (kanamycin resistant) and the donor were also picked and grown up overnight at 37°C in LB liquid medium with appropriate antibiotics. Following the incubation period each bacterial culture was centrifuged at 10,000rpm for 2 minutes. The supernatants were discarded and the pellets resuspended in LB liquid medium. Aliquots of each strain (100μl each) were then mixed and spread with a sterile spreader onto an LB plate with no selection. The plate was inverted and incubated overnight at 27°C in the dark. A loopful of the overnight mating mix was then streaked onto a LB plate containing selective antibiotics (rifampicin, 100μg/ml and kanamycin 50μg/ml). The plate was inverted and incubated for 48-72 hours at 27°C in the dark. Single colonies could then be selected for use in transformation of tomato explants.

Seed sterilisation

Ailsa craig variety of tomato seeds were placed into a sterile 50ml Falcon tube. The seeds were washed with 70% ethanol for 30 seconds and the ethanol removed. 1% Virkon was then added and the tube incubated with shaking at 27°C for 20-30 minutes. 1% Virkon was then added and the tube incubated with shaking at 27°C for 20-30 minutes. The seeds were then washed with sterile dH_2O (~500ml) through a sterile sieve.

Seed sowing

MS3S medium (125ml) was poured per sterile double Magenta pot (Sigma) and allowed to set.

Five sterile seeds were then sown in each pot and incubated for 5 weeks in a control temperature room (27°C) under 5 cool white light tubes with 16 hours photoperiod and 70% relative humidity.

Explant preparation

Plates were prepared for explant preparation by the addition of MS3C5ZR medium to petri dishes (25 plates per litre of medium). A sterile 8.5cm filter disc was then placed onto each plate. Plates were wrapped in cling film and stored at room temperature. Explants were taken under aseptic conditions for 5 week old seedlings. 1-1.5cm sections from above cotyledons were cut and all leaves, roots and leaf nodes were removed. The explants were placed on a filter disc on pre-incubation medium (10 per plate as prepared in step 1. The plates were then sealed and stored at 26°C with low light intensity.

A. tumefaciens culture preparation

Several *A. tumefaciens* colonies from triparental mating containing either pVB6_TSEC-LML or pVB6_35S_TSEC-LML were picked and used to inoculate LB liquid medium (10mls) containing kanamycin (50μg/ml). The culture was incubated in a shaking incubator (225-250rpm) for 24 hours at 27°C. The overnight culture (10mls) was added to LB liquid medium (50mls) containing kanamycin (50μg/ml). This second culture was then incubated for 24 hours at 27°C in a shaking incubator (225-250rpm).

The *A. tumefaciens* culture (40mls) was then briefly centrifuged in a bench-top centrifuge (up to 3,000rpm) to remove clumps of growth. The supernatant was then carefully collected into a sterile 50ml Falcon tube. The supernatant was spun at 3,000rpm in a bench-top centrifuge for 10 minutes and the supernatant discarded. The pellet was resuspended in MS3S (30mls) by vortexing. The culture was diluted to 1/10th with MS3S and the optical density (OD) at 550nm measured with MS3S as a blank. The OD was adjusted to 0.1 with MS3S 20-25mls of culture was prepared for every 50 explants transformed.

Transformation of explants

50 explants were prepared as above (5 plates) and were transferred into petri dishes and 25ml of *A. tumefaciens* solution per petri dish poured over them. They were then incubated at room temperature for 10 minutes before being transferred to petri dishes containing a double layer of sterile filter paper. The explants were then transferred to plates

containing MS3SC5ZR medium (10 per plate). The plates were sealed and then incubated in a control temperature room (27°C) for 48 hours.

Selection

The explants were transferred to selection media MS3C5RCK (10 explants per plate) and sealed before returning to the control temperature room for 2 weeks.

Subculture of explants

Following selection explants were subcultured every 2 weeks on MS3C5ZRCK medium. When shoots developed they were carefully excised and transferred to Phytatrays (Sigma) containing MS3C5CK. DNA samples for PCR analysis were collected when shoots were sufficiently developed. Once the shoots rooted they were transferred to the glasshouse where initially they were placed in vermiculite with 1g/L Osmocote slow release fertiliser and then once roots were established they were transferred to soil.

Constructs for transformation

pVB6_35S-TSEC-LML and pVB6-TSEC-LML are shown in diagrammatic form in Figures 7 and 6 respectively.

Analysis of transformants

1. All transformation were tested for the transgene, using PCR with *E.coli* Dxps-specific primers:

Forward: 5'-GCG CCG CTA TTT ACT CGA-3'

Reverse: 5'TTT CTC TGG CGT GCC GCC-3'

2. Those that were PCR positive were tested by Southern blot analysis for the number of inserts, using the nptII probe.

3. Single insert transformation were then analysed for Dxps expression using RT-PCR, and the primers described in 1, above.

4. Expressing lines were tested for DXPS protein levels using Western blots with an antibody specific for the *E.coli* protein. A band ca. 69 kDa was found, showing both expression of transgene and cleavage of the transit peptide from the mature protein.

5. Seed was collected from all single insert lines for sowing.

6. T1 progeny were cultivated for pigment analysis and inheritance of phenotype.

Isoprenoids constitute a large group of compounds many of which are of high economic value. The condensation of (hydroxy)thiamin, derived from the decarboxylation of pyruvate, with glyceraldehyde-3-phosphate to yield 1-deoxy-D- xylulose-5-phosphate, is considered to be the first reaction in the mevalonate-independent pathway for IPP and ultimately isoprenoid biosynthesis. The data presented show that increasing the rate of DXP synthesis in *E. coli* results in increased isoprenoid biosynthesis. This finding can therefore be utilised to optimise the industrial production of isoprenoids from bacteria. The

manipulation of enzyme activities important in the biosynthesis of specific isoprenoids in concert with DXPS may be employed to bioengineer the production of specific, high value isoprenoids in *E. coli* or another suitable cell or organism such as in plants where increased isoprenoid production could be used for improving crop flavour, fragrance and colour. Alternatively, crops could be engineered to produce increased concentrations of isoprenoids with pharmaceutical and/or nutritional properties.

TABLE 1. DXP synthase activity in *E. coli homogenates*

	Specific activity nmol/min/mg protein	**Fold increase in activity**
Control	5.8 ± 0.07	1.0
B. subtilis	11.5 ± 0.58	2.0
Syn. sp. 6803	10.4 ± 0.24	1.8

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Claims

1. A method of manipulating isoprenoid expression in a plant or plant cell having a mevalonate independent isopentyl diphosphate synthesising pathway, which method comprises altering the activity of the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), or a functional equivalent thereof.

2. A method according to claim 1 wherein said isoprenoid production is increased by enhancing the activity or expression of said DXPS or lowered by inhibiting the activity or expression of said DXPS enzyme.

3. A method according to claim 2 wherein said enhanced DXPS activity occurs by transformation of said plant or plant cell with a vector comprising a nucleic acid molecule encoding said DXPS operably linked to an expression control sequence and optionally a reporter molecule

4. A method according to claim 3 wherein said DXPS encoded by said nucleic acid sequence is endogenous to said plant or plant cell.

5. A method according to claim 3 or 4 wherein said vector comprises one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a desired isoprenoid.

6. A method according to claim 3 or 4 wherein said plant or plant cell is transformed with a further vector comprising one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a

desired isoprenoid.

7. A method according to any of claims 3 to 6 wherein said vector comprising said nucleic acid sequence(s) encoding said DXPS and/or said polypeptide(s) capable of producing said isoprenoid further comprises a nucleic acid sequence of either a tissue specific promoter and/or encoding a plastid transit peptide.

8. A method according to any of claims 5 to 7 wherein said desired isoprenoid is one conferring a nutritional benefit.

9. A method according to claim 8 wherein said isoprenoid comprises any of the carotenoids, vitamins E, B1 or B6, chlorophylls, phenylquinones or diterpenes.

10. A plant or plant cell which has a mevalonate independent IPP biosynthetic pathway and which is transformed or transfected with a vector comprising a nucleic acid sequence encoding DXPS or a functional equivalent, derivative or bioprecursor thereof operably linked to an expression control sequence.

11. A plant or plant cell according to claim 10 wherein said vector further comprises a nucleic acid molecule encoding a reporter molecule.

12. A plant or plant cell according to claim 10 or 11 which further comprises a vector comprising one or more nucleic acid sequences encoding one or more polypeptides capable of producing a desired isoprenoid.

13. A plant or plant cell according to claim 12 wherein said desired isoprenoid comprises any of the carotenoids, vitamin E, B1 or B6, chlorophylls, phenylquinones, or diterpenes.

14. A method of manipulating isoprenoid expression in a cell or organism having a mevalonate independent isopentyl diphosphate synthesising pathway, which method comprises altering the activity of the enzyme 3-deoxy-D-xylulose-5-phosphate synthase (DXPS) or a functional equivalent thereof by transforming said cell or organism with a vector comprising a nucleic acid optionally linked to an expression control sequence and operably a reporter molecule, and a further vector comprising one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a desired isoprenoid.

15. A method according to claim 14, wherein said nucleic acid sequence encoding said DXPS is endogenous to said cell or organism.

16. A method according to claim 14 or 15, wherein said cell is any of a bacterial, yeast or algal cell.

17. A method according to claim 16, wherein said bacterial cell is *E. coli*.

18. A method according to claim 14 or 15 wherein said organism is a plant.

19. A method according to any of claims 14 to 18 wherein said cell is any of a bacterial, yeast or algal cell.

20. A method according to any of claims 14 to 19

wherein said bacterial cell is *E. coli*.

21. A method according to claim 20 wherein said organism is a plant.

22. A method of identifying a compound which modulates isoprenoid activity or expression, said method comprising contacting said compound to be tested with a molecule which is a component of the mevalonate independent IPP biosynthetic pathway and which molecule undergoes a reaction catalysed by DXPS activity in the presence of an appropriate reactant, in the presence of DXPS or a functional equivalent thereof and monitoring the yield of a product of the reaction when compared to the same reaction performed in the absence of the compound to be tested.

23. A method according to claim 22 wherein said molecule comprises pyruvate and said appropriate reactant comprises glyceraldehyde-3-phosphate or vice versa.

24. A transgenic cell, tissue or organism having a mevalonate independent IPP biosynthetic pathway and increased isoprenoid activity which cell, tissue or organism comprises at least one transgene capable of expressing DXPS or a functional equivalent thereof.

25. A transgenic cell, tissue or organism according to claim 24, which comprises at least one additional copy of any of the nucleic acid sequences identified in Figure 3, or the complement thereof.

26. A transgenic cell, tissue or organism according to claim 24 or 25, further comprising a transgene capable of expressing one or more polypeptides capable of producing a desired

isoprenoid, or a functional equivalent.

27. A transgenic cell, tissue or organism according to any of claims 24 to 26, wherein said organism is a plant.

28. A transgenic cell tissue or organism according to claim 27, wherein said plant is of the Lycopersicon spp.

29. Progeny of the organism according to any of claims 24 to 28 having increased isoprenoid activity.

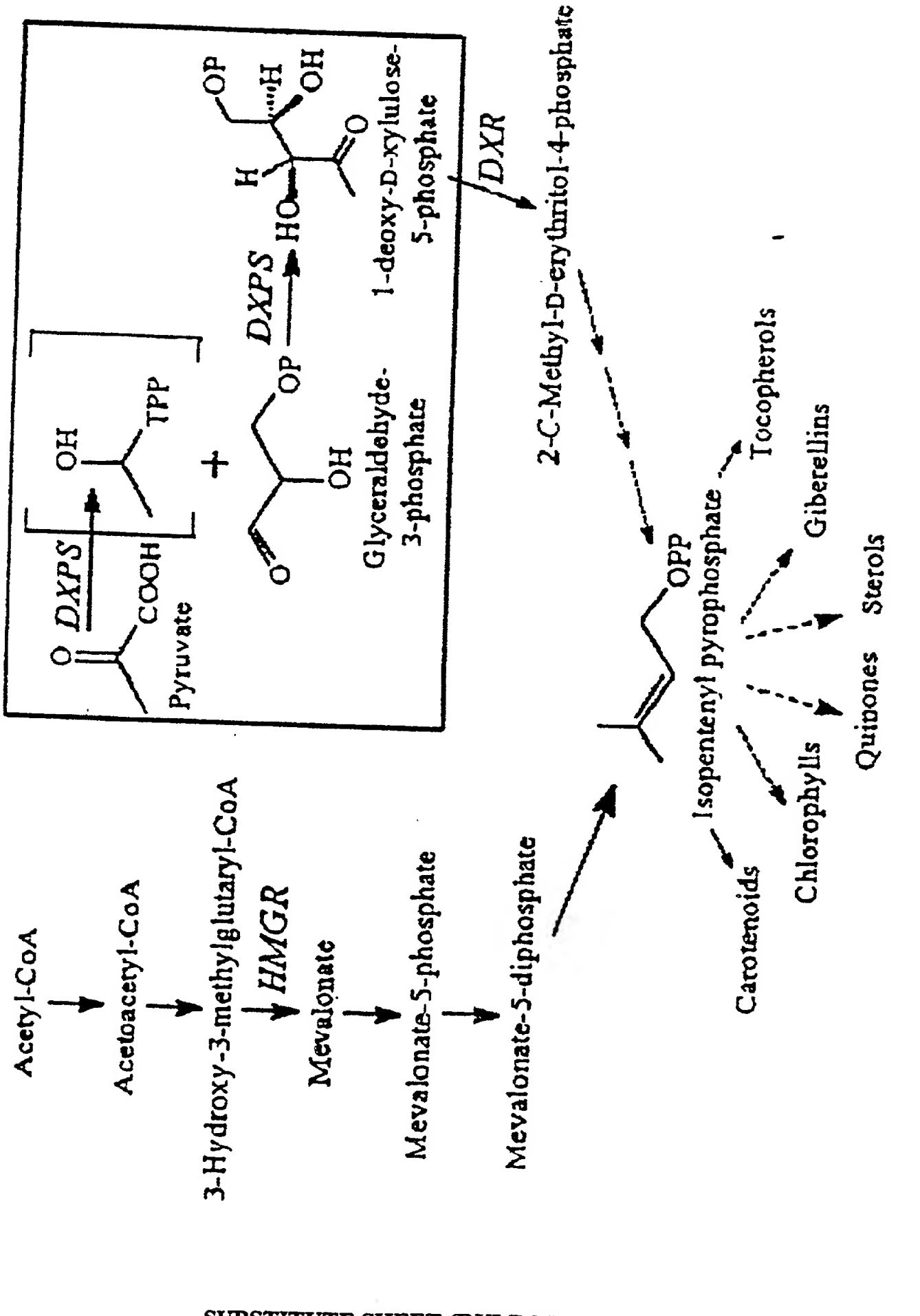
30. A transformed plant comprising a transgene capable of expressing DXPS from *E.coli* having the sequence according to Figure 8 and which plant comprises a higher level of isoprenoid than an untransformed plant.

31. A transformed plant according to claim 30 comprising any of constructs pVB6_TSEC_LML or pVB6_35S_TSEC-LML illustrated in Figures 6 and 7.

32. A transformed plant according to claim 30 or 31 wherein said plant is a tomato plant.

33. A tomato fruit produced by a plant according to claim 32 and having a higher level of isoprenoid activity than a wild type fruit.

34. A seed produced by a plant according to claim 32 and having a higher level of isoprenoid activity than a seed from a wild type plant.


B
DXPS
TPP
OH
COOH
Pyruvate
+
DXPS
OP
HO
H
OH
O
OP
O
OH
Glyceraldehyde-3-phosphate
1-deoxy-D-xylulose-5-phosphate
DXR
2-C-Methyl-D-erythritol-4-phosphate
OPP
Isopentenyl pyrophosphate
Tocopherols
Giberellins
Sterols
Carotenoids
Chlorophylls
Quinones
A
Acetyl-CoA
Acetoacetyl-CoA
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HMGR
Mevalonate
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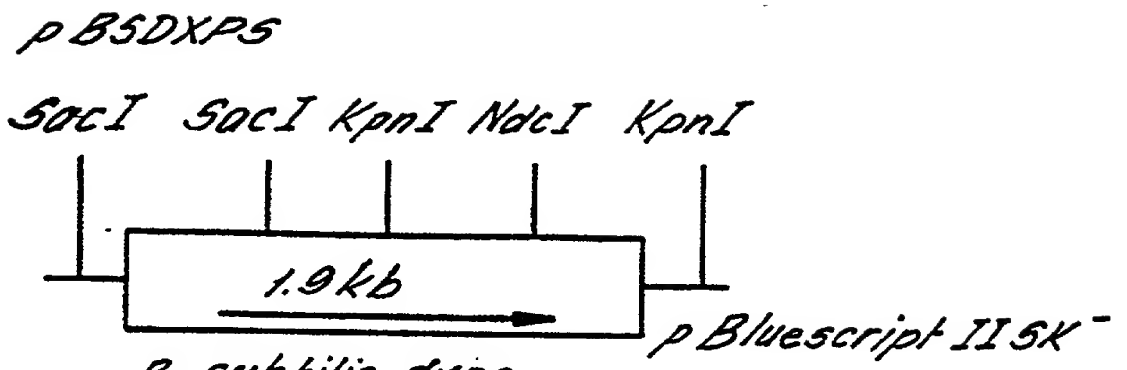
Mevalonate-5-diphosphate

FIG. 1.

FIG. 2.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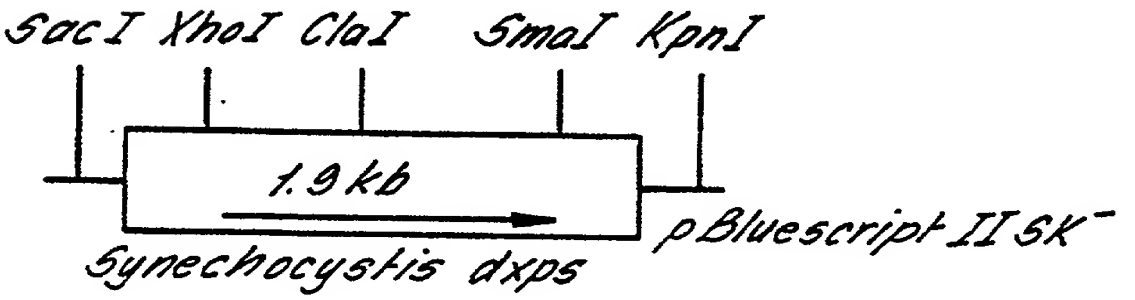
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pBluescript II SK⁻
Synechocystis dxps

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bacillus  ---------MDLLSIQDPSFLKNMSIDELEKLSDEIRQELITSLSASGGHIGPNLGVVELT
Ecolix0   MSFDIAKYPTLALVDSTQELRLLPKESLPKLCDELRRYLLDSVSRSSGHFASGLGTVELT

synxxx1   VALHKEFNSPKDKFLWDVGHQSYVHKLLTGRGKEFATLRQYKGLCGFPKRSESEHDVWET
bacillus  VALHKEFNSPKDKFLWDVGHQSYVHKLLTGRGKEFATLRQYKGLCGFPKRSESEHDVWET
Ecolix0   VALHYVYNTPFDQLIWDVGHQAYPHKILTGRRDKIGTIRQKGGLHPFPWRGESEYDVLSV

synxxx1   GHSSTSLSGAMGMAAARDIKGTDEYIIPIIGDGALTGGMALEALNHIGDEKKDMIVILND
bacillus  GHSSTSLSGAMGMAAARDIKGTDEYIIPIIGDGALTGGMALEALNHIGDEKKDMIVILND
Ecolix0   GHSSTSISAGIGIAVAAEKEGKNRRTVCVIGDGAITAGMAFEAMNHAGDIRPDMLVILND

synxxx1   NEMSIAPNVGAIHSMLGRLRTAGKYQWVKDELEYLFKKIPAVGGKLAATAERVKDSLKYM
bacillus  NEMSIAPNVGAIHSMLGRLRTAGKYQWVKDELEYLFKKIPAVGGKLAATAERVKDSLKYM
Ecolix0   NEMSISENVGALNNHLAQLLSGKLYSSLREGGKKVFSGVPPIKELLKRTEEHIKG----M

synxxx1   LVSGMFFEELGFTYLGPVDGHSYHELIENLQYAKKTKGPVLLHVITKKGKGYKPAETDTI
bacillus  LVSGMFFEELGFTYLGPVDGHSYHELIENLQYAKKTKGPVLLHVITKKGKGYKPAETDTI
Ecolix0   VVPGTLFEELGFNYIGPVDGHDVLGLITTLKNMRDLKGPQFLHIMTKKGRGYEPAEKDPI

synxxx1   GTWHGTGPYKINTGDFVKPKAAAPSWSGLVSGTVQRMAREDGRIVAITPAMPVGSKLEGF
bacillus  GTWHGTGPYKINTGDFVKPKAAAPSWSGLVSGTVQRMAREDGRIVAITPAMPVGSKLEGF
Ecolix0   -TFHAVPKFDPSSGCLPKSSGGLPSYSKIFGDWLCETAAKDNKLMAITPAMREGSGMVEF

synxxx1   AKEFPDRMFDVGIAEQHAATMAAAMAMQGMKPFLAIYSTFLQRAYDQVVHDICRQNANVF
bacillus  AKEFPDRMFDVGIAEQHAATMAAAMAMQGMKPFLAIYSTFLQRAYDQVVHDICRQNANVF
Ecolix0   SRKFPDRYFDVAIAEQHAVTFAAGLAIGGYKPIVAIYSTFLQRAYDQVLHDVAIQKLPVL

synxxx1   IGIDRAGLVGADGETHQGVFDIAFMRHIPNMVLMMPKDENEGQHMVHTALSYDEGPIAMR
bacillus  IGIDRAGLVGADGETHQGVFDIAFMRHIPNMVLMMPKDENEGQHMVHTALSYDEGPIAMR
Ecolix0   FAIDRAGIVGADGQTHQGAFDLSYLRCIPEMVIMTPSDENECRQMLYTGYHYNDGPSAVR

synxxx1   FPRGNGLGVKMDEQLKTIPIGTWEVLRPGNDAVILTFGTTIEMAIEAAEELQKEGLSVRV
bacillus  FPRGNGLGVKMDEQLKTIPIGTWEVLRPGNDAVILTFGTTIEMAIEAAEELQKEGLSVRV
Ecolix0   YPRGNAVGVELTP-LEKLPIGKGIVKRRGEKLAILNFGTLMPEAAKVAESLN-----ATL

synxxx1   VNAREIKPIDEKMMKSILKEGLPILTIEEAVLEGGFGSSILEFAHDQGEYHTPIDRMGIP
bacillus  VNAREIKPIDEKMMKSILKEGLPILTIEEAVLEGGFGSSILEFAHDQGEYHTPIDRMGIP
Ecolix0   VDMRFVKPLDEALILEMAASHEALVTVEENAIMGGAGSGVNEVLMAHR-KPVPVLNIGLP

synxxx1   DRFIEHGSVTALLEEIGLTKQQVANRIRLLMPPKTHKGIGS
bacillus  DRFIEHGSVTALLEEIGLTKQQVANRIRLLMPPKTHKGIGS
Ecolix0   DFFIPQGTQEEMRAELGLDAAGMEAKIKAWLA----------
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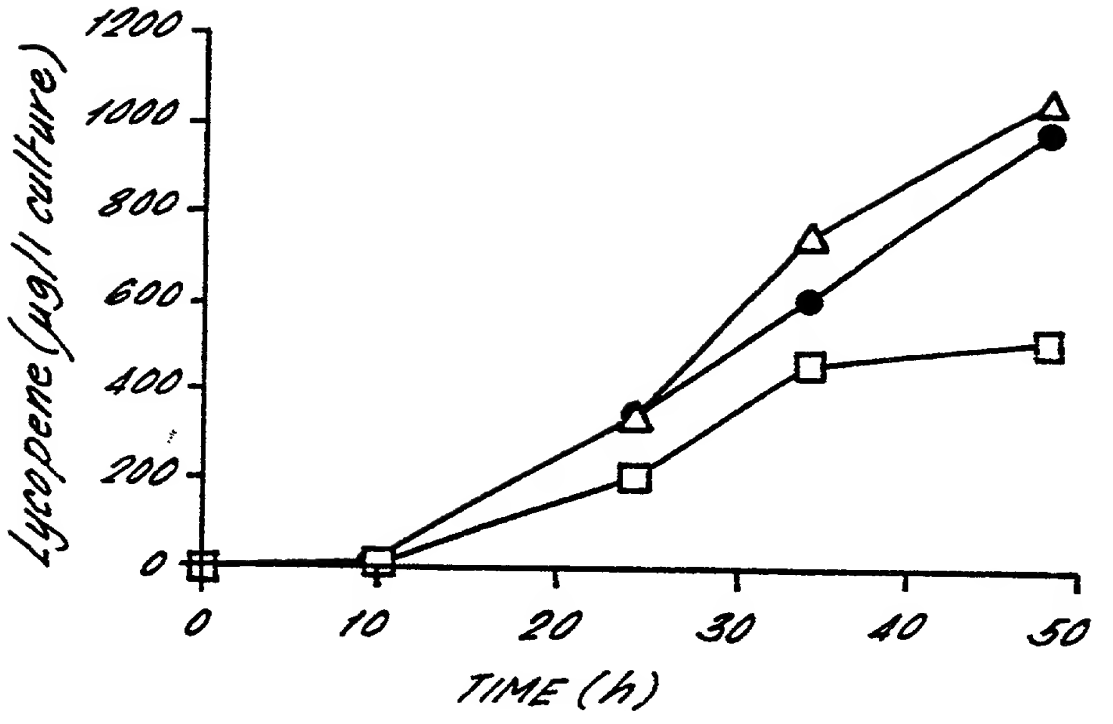
FIG. 3.

FIG. 4.


Lycopene (µg/l culture)
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1000
800
600
400
200
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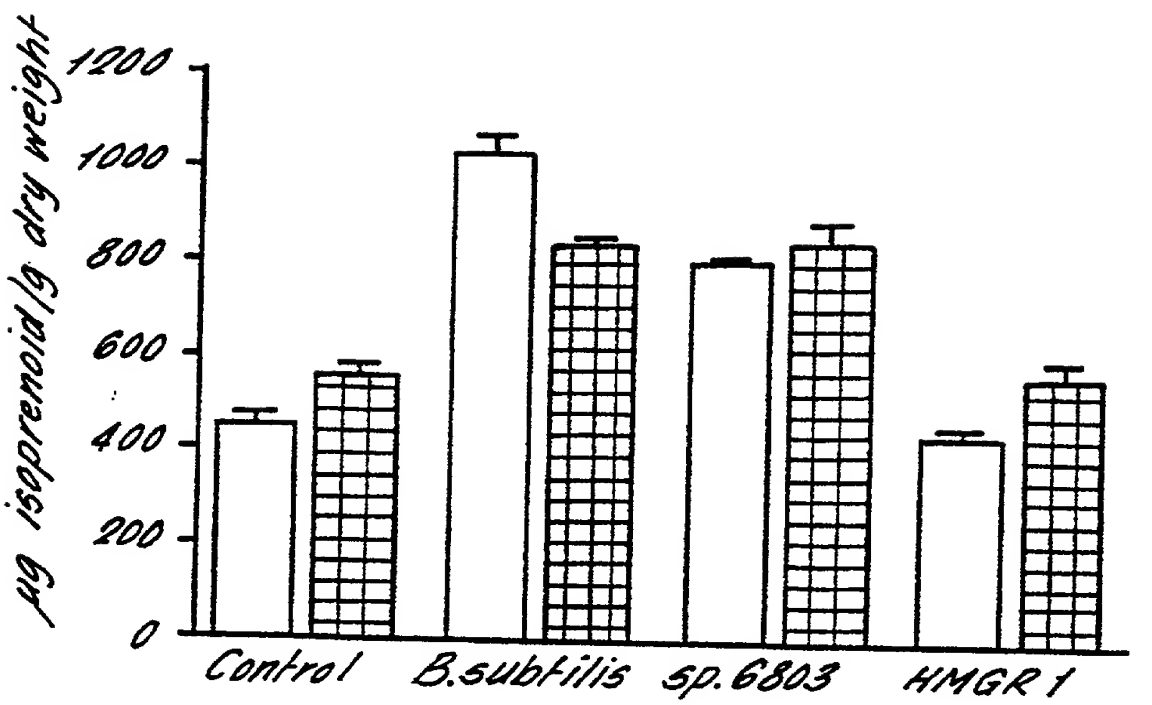
0
10
20
30
40
50
TIME (h)

FIG. 5.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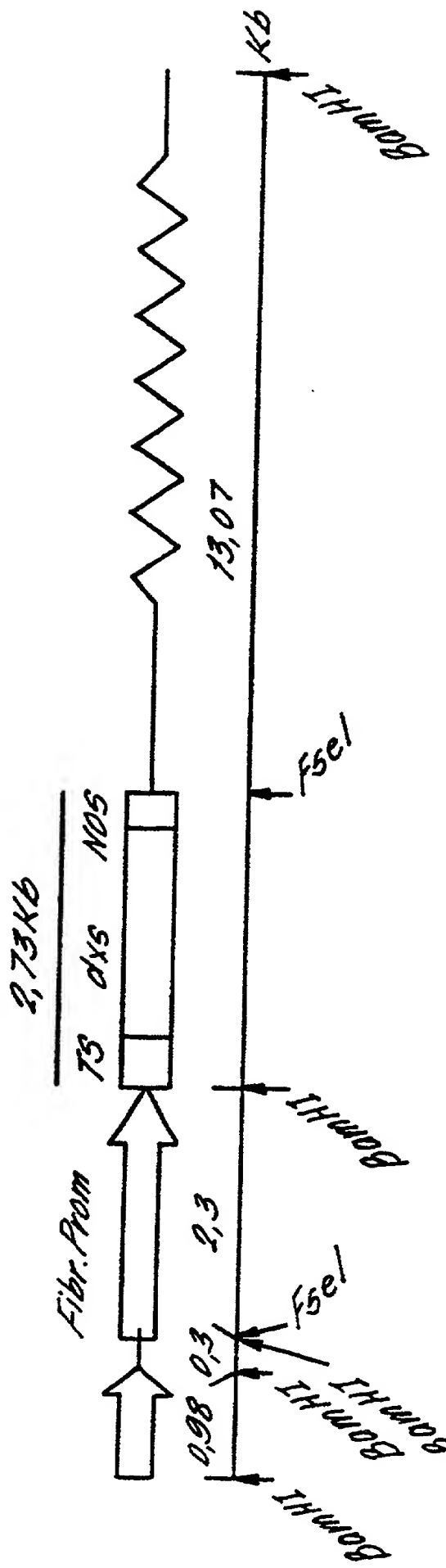
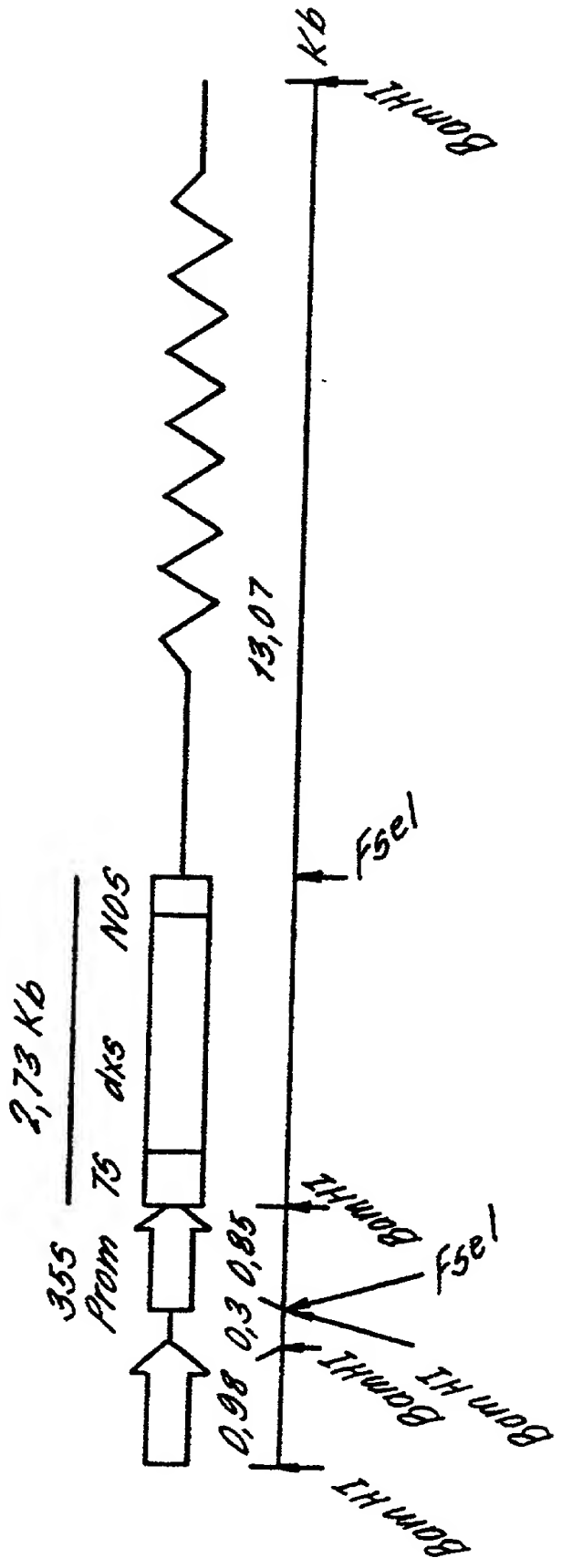


FIG. 6.

Construction name: pVB6_TSEC_LML
Fibr.Prom: Fibrilin promoter
TS: transit sequence
dXS: deoxyxylulose synthase gene from E. coli
Date: October 1998

7/8

FIG. 7.



Construction name: pVBC_355_TSEC_LML
355 Prom: 355 promoter
TS: transit sequence
dxs: deoxyxylulose synthase gene from E.coli
Date: October 1998

FIG. 8.

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yphkiltgrrdkigtirqkgglhpfpwrgeseydvlsvghsstsisagigiavaaekegknrrtvcvigdgaitagmafe
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FIG. 9.

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tlrqtdglagftkrseseydcfg

Attorney Docket No. B0192/7031

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled

MANIPULATING ISOPRENOID EXPRESSION

the specification of which is attached hereto unless the following is checked:

[X] was filed on July 27, 2001, as United States Application No. 09/890,229, bearing attorney docket no. B0192/7031, and was amended on July 27, 2001.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or section 365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed:

Prior Foreign PCT International Application(s)and any priority claims under 35 U.S.C. §§119 and 365(a),(b):

			Priority Claimed	
9901902.8 (Number)	**Great Britain** (Country-if PCT, so indicate)	**January 28, 1999** (DD/MM/YY Filed)	**[X]** YES	[ ] NO
(Number)	(Country-if PCT, so indicate)	(DD/MM/YY Filed)	[ ] YES	[ ] NO
(Number)	(Country-if PCT, so indicate)	(DD/MM/YY Filed)	[ ] YES	[ ] NO

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

(Application Number)	(filing date)
(Application Number)	(filing date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application No.)	(filing date)	(status-patented, pending, abandoned)
(Application No.)	(filing date)	(status-patented, pending, abandoned)

PCT International Applications designating the United States:

(PCT Appl. No.)	(U.S. Ser. No.)	(PCT filing date)	(status-patented,pending,abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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John R. Van Amsterdam
c/o Wolf, Greenfield & Sacks, P.C.,
Federal Reserve Plaza
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Boston, MA 02210-2211

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment,

or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Peter M Bramley 16/10/07

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Citizenship: Great Britain
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SEQUENCE LISTING

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Harker, Mark

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Thr Gly Arg Tyr His Asp Phe His Thr Leu Arg Gln Lys Asp Gly Val
85 90 95

Ala Gly Tyr Leu Lys Arg Ser Glu Ser Arg Phe Asp His Phe Gly Ala
100 105 110

Gly His Ala Ser Thr Ser Ile Ser Ala Gly Leu Gly Met Ala Leu Ala
115 120 125

Arg Asp Ala Lys Gly Glu Asp Phe Lys Val Val Ser Ile Ile Gly Asp
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Gly Ala Leu Thr Gly Gly Met Ala Leu Glu Ala Ile Asn His Ala Gly
145 150 155 160

His Leu Pro His Thr Arg Leu Met Val Ile Leu Asn Asp Asn Glu Met
165 170 175

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Ile Lys His Leu Pro Phe Val Gly Asp Ser Leu Thr Pro Glu Met Glu
    210                 215                 220

Arg Val Lys Glu Gly Met Lys Arg Leu Val Val Pro Lys Val Gly Ala
225                 230                 235                 240

Val Ile Glu Glu Leu Gly Phe Lys Tyr Phe Gly Pro Ile Asp Gly His
                245                 250                 255

Ser Leu Gln Glu Leu Ile Asp Thr Phe Lys Gln Ala Glu Lys Val Pro
            260                 265                 270

Gly Pro Val Phe Val His Val Ser Thr Thr Lys Gly Lys Gly Tyr Asp
        275                 280                 285

Leu Ala Glu Lys Asp Gln Val Gly Tyr His Ala Gln Ser Pro Phe Asn
    290                 295                 300

Leu Ser Thr Gly Lys Ala Tyr Pro Ser Ser Lys Pro Lys Pro Pro Ser
305                 310                 315                 320

Tyr Ser Lys Val Phe Ala His Thr Leu Thr Thr Leu Ala Lys Glu Asn
                325                 330                 335

Pro Asn Ile Val Gly Ile Thr Ala Ala Met Ala Thr Gly Thr Gly Leu
            340                 345                 350

Asp Lys Leu Gln Ala Lys Leu Pro Lys Gln Tyr Val Asp Val Gly Ile
        355                 360                 365

Ala Glu Gln His Ala Val Thr Leu Ala Ala Gly Met Ala Cys Glu Gly
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Ile Arg Pro Val Val Ala Ile Tyr Ser Thr Phe Leu Gln Arg Gly Tyr
385                 390                 395                 400

Asp Gln Ile Ile His Asp Val Cys Ile Gln Lys Leu Pro Val Phe Phe
                405                 410                 415

Cys Leu Asp Arg Ala Gly Ile Val Gly Ala Asp Gly Pro Thr His Gln
            420                 425                 430

Gly Met Tyr Asp Ile Ala Tyr Leu Arg Cys Ile Pro Asn Leu Val Leu
        435                 440                 445

Met Ala Pro Lys Asp Glu Ala Glu Leu Gln Gln Met Leu Val Thr Gly
    450                 455                 460

Val Asn Tyr Thr Gly Gly Ala Ile Ala Met Arg Tyr Pro Arg Gly Asn
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Ala Glu Ala Leu Met Asp Asn Asn Val Leu Val Pro Leu Lys Arg Leu
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Gly Val Pro Asp Ile Leu Val Asp His Ala Thr Pro Glu Gln Ser Thr
        595                 600                 605

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Thr Ser Leu Ser Ala Ser Gly Gly His Ile Gly Pro Asn Leu Gly Val
        35                  40                  45

Val Glu Leu Thr Val Ala Leu His Lys Glu Phe Asn Ser Pro Lys Asp
    50                  55                  60

Lys Phe Leu Trp Asp Val Gly His Gln Ser Tyr Val His Lys Leu Leu
65                  70                  75                  80

Thr Gly Arg Gly Lys Glu Phe Ala Thr Leu Arg Gln Tyr Lys Gly Leu
                85                  90                  95

Cys Gly Phe Pro Lys Arg Ser Glu Ser Glu His Asp Val Trp Glu Thr
            100                 105                 110

Gly His Ser Ser Thr Ser Leu Ser Gly Ala Met Gly Met Ala Ala Ala
        115                 120                 125
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Arg Asp Ile Lys Gly Thr Asp Glu Tyr Ile Ile Pro Ile Ile Gly Asp
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Gly Ala Leu Thr Gly Gly Met Ala Leu Glu Ala Leu Asn His Ile Gly
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Asp Glu Lys Lys Asp Met Ile Val Ile Leu Asn Asp Asn Glu Met Ser
                165                 170                 175

Ile Ala Pro Asn Val Gly Ala Ile His Ser Met Leu Gly Arg Leu Arg
            180                 185                 190

Thr Ala Gly Lys Tyr Gln Trp Val Lys Asp Glu Leu Glu Tyr Leu Phe
        195                 200                 205

Lys Lys Ile Pro Ala Val Gly Gly Lys Leu Ala Ala Thr Ala Glu Arg
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Leu Leu His Val Ile Thr Lys Lys Gly Lys Gly Tyr Lys Pro Ala Glu
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Thr Asp Thr Ile Gly Thr Trp His Gly Thr Gly Pro Tyr Lys Ile Asn
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Thr Gly Asp Phe Val Lys Pro Lys Ala Ala Ala Pro Ser Trp Ser Gly
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Leu Val Ser Gly Thr Val Gln Arg Met Ala Arg Glu Asp Gly Arg Ile
                325                 330                 335

Val Ala Ile Thr Pro Ala Met Pro Val Gly Ser Lys Leu Glu Gly Phe
            340                 345                 350

Ala Lys Glu Phe Pro Asp Arg Met Phe Asp Val Gly Ile Ala Glu Gln
        355                 360                 365

His Ala Ala Thr Met Ala Ala Ala Met Ala Met Gln Gly Met Lys Pro
    370                 375                 380

Phe Leu Ala Ile Tyr Ser Thr Phe Leu Gln Arg Ala Tyr Asp Gln Val
385                 390                 395                 400

Val His Asp Ile Cys Arg Gln Asn Ala Asn Val Phe Ile Gly Ile Asp
                405                 410                 415

Arg Ala Gly Leu Val Gly Ala Asp Gly Glu Thr His Gln Gly Val Phe
            420                 425                 430

Asp Ile Ala Phe Met Arg His Ile Pro Asn Met Val Leu Met Met Pro
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Lys Asp Glu Asn Glu Gly Gln His Met Val His Thr Ala Leu Ser Tyr
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His Gln Ser Tyr Pro His Lys Ile Leu Thr Gly Arg Arg Asp Lys Met
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